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Award Number:
W81XWH-08-1-0200

TITLE:
Activation of Estrogen Receptor-alpha by Novel Anions.

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CONTRACTING ORGANIZATION:
Georgetown University

Washington, DC 20057

REPORT DATE:
April 2010

TYPE OF REPORT:
Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-04-2010	Annual Summary	5 March 2009 – 4 March 2010

4. TITLE AND SUBTITLE
Activation of Estrogen Receptor-alpha by novel anions.

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0200

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Geoffrey Storchan

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Georgetown University

Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical and Materiel Command
Fort Detrick, MD
21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The overall purpose of my project is to investigate the direct interaction of ER-alpha with eNOS and nitrite, and to determine what roles they might play in hormone independent breast cancers. I hope to characterize the interaction between ER-alpha and eNOS in the absence and presence of growth factors, determine whether polymorphic forms of eNOS increase ER-alpha activity due to increased nitrite production, and to determine the mechanism by which nitrite activates ER-alpha. Important findings to date have shown that treatment of wild-type ER-alpha with nitrite leads to the dissociation of the hsp90 complex, binding to DNA, and the recruitment of the coactivator SRC-1 and RNA polymerase II to estrogen regulated gene promoters. These findings provide a novel role for nitrite in activation of ER-alpha.

15. SUBJECT TERMS
estrogen receptor-alpha, anions, nitric oxide synthase, mechanisms, metalloestrogens

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	12	19b. TELEPHONE NUMBER *(include area code)*
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Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Our lab has shown that treatment of MCF-7 breast cancer cells with growth factor leads to the activation of the Akt pathway, and subsequent activation of endothelial nitric oxide synthase (eNOS). Treatment of MCF-7 cells with IGF-1 led to an increase in the expression of estrogen regulated genes that was blocked by eNOS inhibitor L-NAME and the anti-estrogen ICI 182,720. To define the pathway by which IGF-1 activates ER-alpha, we stably transfected constitutively activated AKT into MCF-7 cells. Activation of this pathway again led to an increase in estrogen regulated genes, and was blocked by L-NAME and ICI 182,720. Finally, we tested the ability of nitrite to activate ER-alpha. Treatment of MCF-7 cells with nitrite led to an increase in growth and estrogen regulated genes, which was blocked by ICI 182,720, suggesting that nitrite activates ER-alpha. In addition, mutational analysis of ER-alpha revealed six amino acids within the ligand binding domain of the receptor that are important in the activation of ER-alpha by nitrite (1). Determining the mechanism by which nitrite activates ER-alpha will provide valuable information for future treatment of breast cancer. The overall goal of my project is to investigate the interaction of ER-alpha with eNOS and nitrite, and what role they might play in hormone independent breast cancers.

Body

Objective 1: To characterize the physiological interaction of ER-alpha with eNOS (*18 months*).

Antibodies for ER-alpha and eNOS have been selected. Protocol for coimmunoprecipitation has been completed. Objective 1 should be completed within the next 12 months.

Objective 2: Determine whether polymorphic forms of eNOS increase the activity of ER-alpha due to increased synthesis of nitrite (*6 months*).

To date our lab has not obtained any polymorphic forms of eNOS in order to test their ability to increase nitrite levels and ER-alpha activity.

Recently, we have been able to determine nitrite levels in MCF-7 cells in the presence and/or absence of IGF-1 and the eNOS inhibitor N^G-nitro-L-arginine methyl ester HCl (L-NAME) using a nitrate/nitrite fluorometric kit (Cayman Chemical). I am currently repeating this assay and will have final results within three months.

Objective 3: Determine the mechanism by which nitrite activates the estrogen receptor-alpha *(36 months).*

Our lab has shown that nitrite is able to interact with specific amino acids located on helices H4, H10, H11, and H12 of the ligand binding domain of the receptor. Molecular modeling suggest that lys529 on H11 and asn532 on the loop between H11 and H12 form site A of interaction, his516 on H10 and lys520 on H11 form site B, and

cys381 on H4 and his547 on H12 form the final site C of the interaction (Figure 1). I predict that the interaction of nitrite with site A is necessary for the initial movement of helix H12 over the ligand binding pocket of the receptor allowing for disassociation from the hsp90 complex. Therefore, mutants of site A will remain associated with hsp90 upon treatment with nitrite. Site B is involved in the straightening of helices H10 and H11, creating one continuous helix. This helix makes up a portion of the dimerization domain of the receptor and therefore, site B mutants should dissociate from the hsp90 complex. Finally, the last interaction site is necessary for the proper formation of the coactivator binding site. A shallow hydrophobic groove formed by the positioning of helix H12 under helix H4 and the movement of helix H3 into the proper alignment is necessary for coactivator binding to the receptor. Therefore, site C mutants should dissociate from the hsp90 complex, bind DNA, but not recruit coactivator necessary for transcription. I have tested the ability of wild type and mutant forms of ER-alpha to dissociate from the hsp90 complex, translocate into the nucleus and bind DNA, and recruit SRC-1 and RNA polymerase II to estrogen regulated gene promoters.

Task one has been completed using HEK 293 cells rather than COS 7 cells as mentioned in the pervious annual summary. Briefly, wild type and mutant forms of ER-alpha were transfected into HEK 293 cells, 48 hours post-transfection the cells were either left untreated or treated with 1nM estradiol or 1uM sodium nitrite for three hours. Following treatment the cells were collected, lysed, incubated with antibody to ER-alpha (H-184, Santa Cruz) and immunoblot was performed. All forms of the receptor dissociated hsp90 upon treatment with estradiol as expected. However, upon treatment with nitrite, the mutant receptors lys529 and asn532 failed to dissociate from hsp90 (Figure 2). In addition, as a control, wild-type ER-alpha was tested for its ability to dissociate from hsp70, another protein in the inactive receptor/hsp90 complex, and it too dissociated upon treatment with estradiol and nitrite.

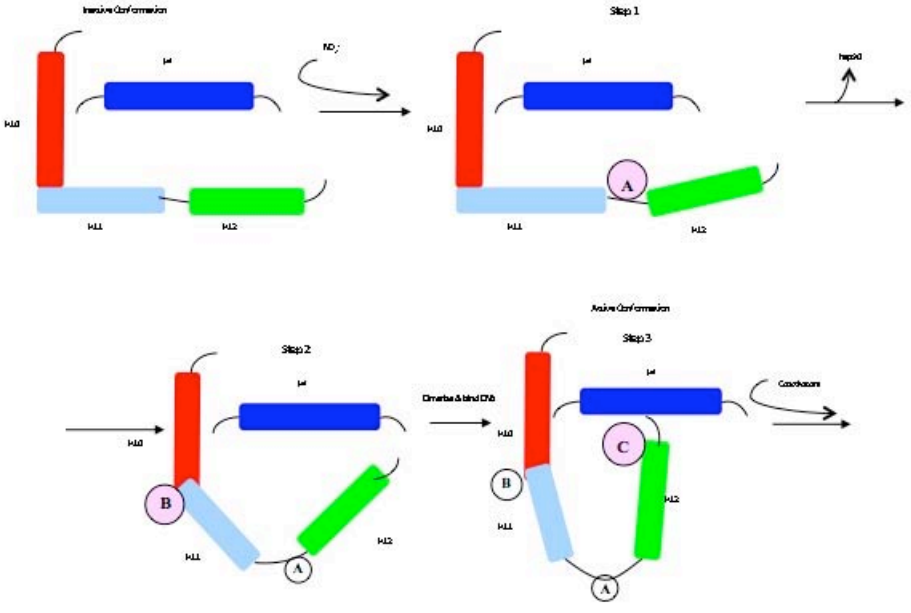

Figure 1. Interaction of nitrite with site A is necessary for the initial movement of helix H12 over the ligand binding pocket and dissociation of hsp90 from the receptor complex. Site B is involved in the straightening of helices H10 and H11 of the ligand binding domain. These helices make up part of the dimerization

domain of the receptor and will be necessary for homodimerization and DNA binding. Site C is critical for the proper formation of the coactivator binding site of the receptor. A shallow hydrophobic groove formed by the repositioning of helix H12 under helix H4 and the movement of helix H3 into the proper alignment is necessary for coactivator binding to the receptor.

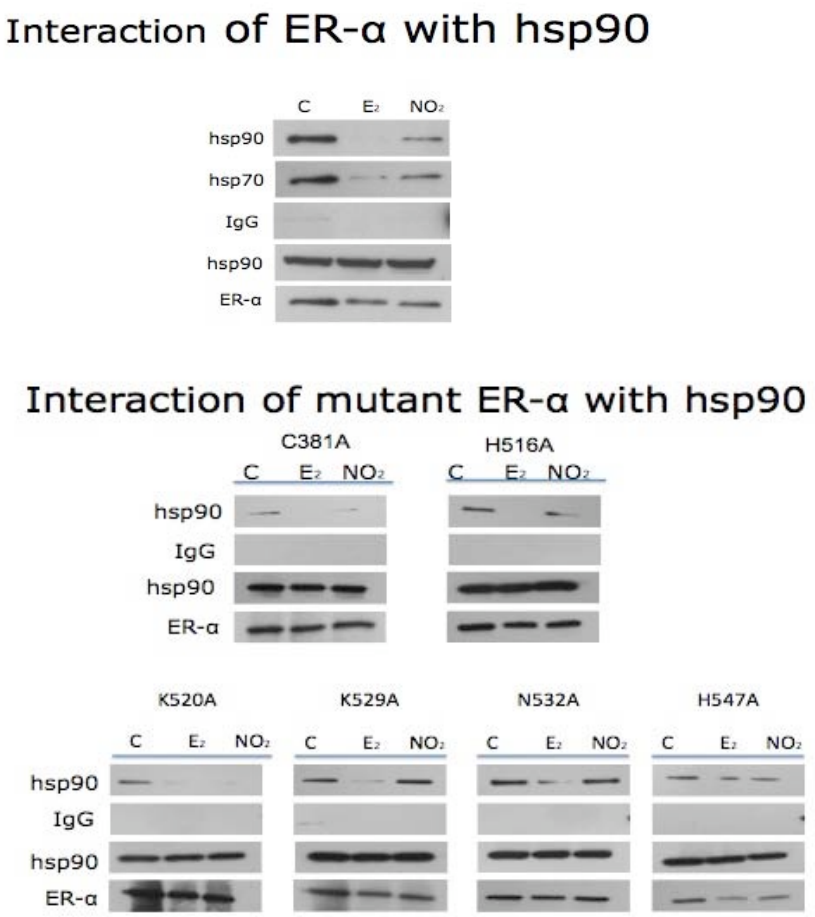

Figure 2. Effects of nitrite on ER-alpha dissociation from hsp90.
Wild type and mutant forms of ER-alpha were transfected into HEK 293 cells and treated with 1nM estradiol or 1uM sodium nitrite for three hours. Cells were collected, lysed, and immunoprecipitated with antibody to ER-alpha. Membranes were blotted with antibodies for ER-alpha (6F11, Vector Labs), hsp90 (AC88, Stressgen), and hsp70 (7/Hsp70, BD Transduction Laboratories).

To accomplish task two within this objective, chromatin immunoprecipitation (ChIP) assays were conducted in HEK 293 cells transfected with ER-alpha. A region of the human complement C3 (hC3) promoter and pS2 promoters containing estrogen response elements (EREs) were amplified by polymerase chain reaction (PCR). Following a three hour treatment with either estradiol or nitrite, wild type and mutant expressing HEK 293 cells were cross-linked with 1% formaldehyde, collected and lysed. Cell lysates were precipitated overnight with antibody to ER-alpha (H-184, Santa Cruz), washed, crosslinks were reversed and PCR was performed. Upon treatment with estradiol, all forms of ER-alpha were recruited to both the hC3 and pS2 promoters (Figure 3). However, upon nitrite treatment only the wild type, lys520 and his547 forms

of the receptor were recruited to the promoters. The inconsistent results seen with asn532 have been resolved and I am able to conclude that it is not recruited to DNA.

In addition to the ChIP assay mentioned above, I am currently working on using immunohistochemistry (IHC) to help visualize the translocation of ER-alpha into the nucleus following treatment with estradiol and nitrite. In addition, I hope to develop coimmunoprecipitation assays using a Gal4/ER-alpha (Gal-ER) fusion protein that will be used to investigate the dimerization of the receptor in the presence of nitrite.

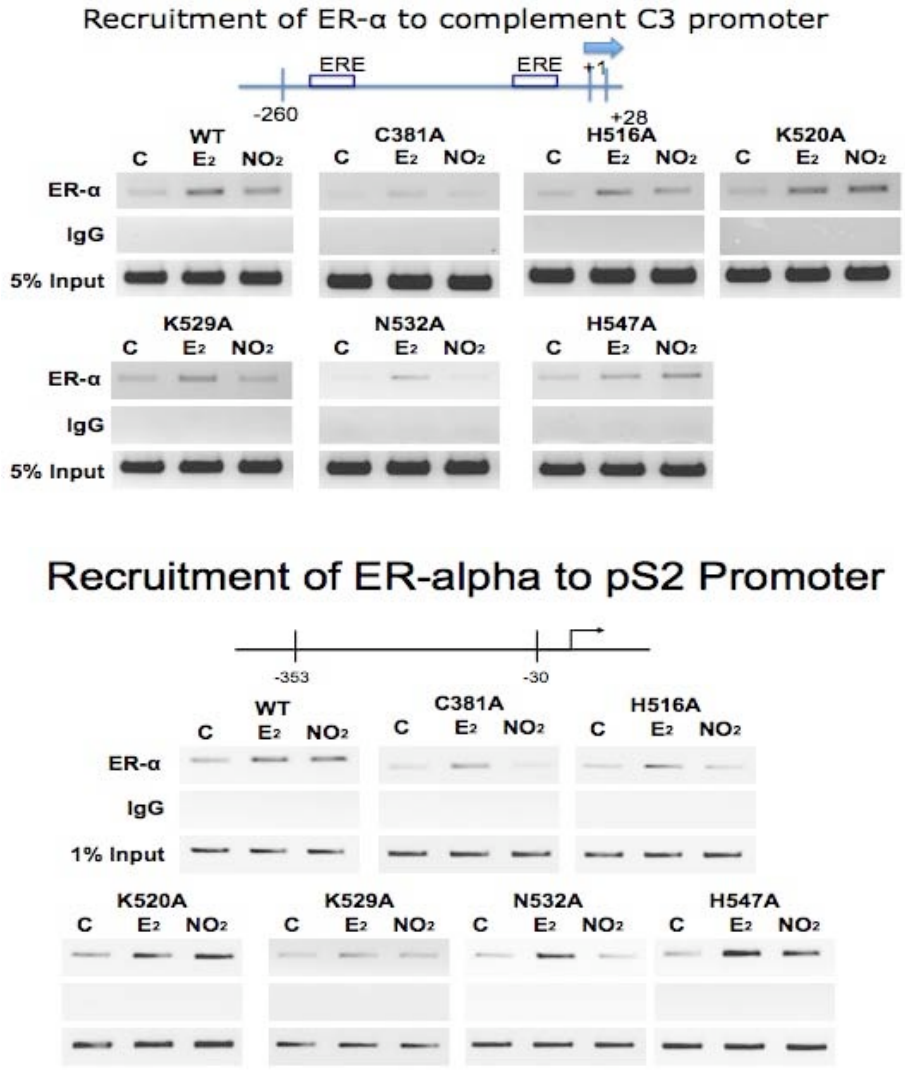

Figure 3. Effects of nitrite on the recruitment of ER-alpha to the hC3 and pS2 promoters. ChIP assays were performed in HEK 293 cells transfected with wild type and mutant ER-alpha. Cells were treated for three hours, cross-linked, collected, and lysed. Following precipitation with antibody to ER-alpha and incubation with protein A beads, the crosslinks were reversed and DNA amplified with primers specific to each region by PCR.

The final task for objective three will be to investigate the interaction of wild type and mutant ER-alpha with coactivator and RNA polymerase II after treatment with nitrite. Re-ChIP assays have been conducted in order to determine if the steroid receptor coactivator SRC-1 and RNA polymerase II have been recruited to the promoter by ER-alpha. Re-ChIP assays were performed as described above for regular ChIP, except that after the first step of immunoprecipitation with antibody to ER-alpha, the captured chromatin was immunoprecipitated a second time with either an antibody to SRC-1 (C-20, Santa Cruz) or with antibody to RNA pol II (CTD4H8, Millipore). Estradiol treatment recruited both SRC-1 and RNA pol II to the hC3 promoter for all mutants, while nitrite only recruited SRC-1 and RNA pol II in the presence of the wild-type receptor (Figure 4 and 5).

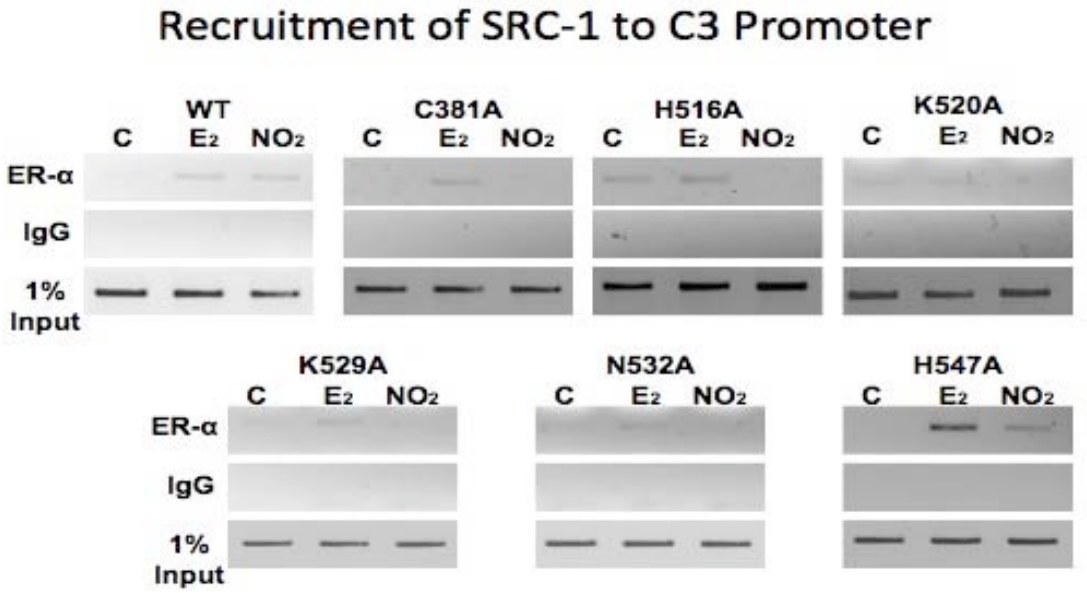

Figure 4. Effects of nitrite on the recruitment of SRC-1 to the hC3 promoter. ChIP assays were conducted as described above, however, following the first immunoprecipitation with ER-alpha antibody a second step of immunoprecipitation was performed with antibody to SRC-1. PCR primers were used to amplify a region of the hC3 promoter.

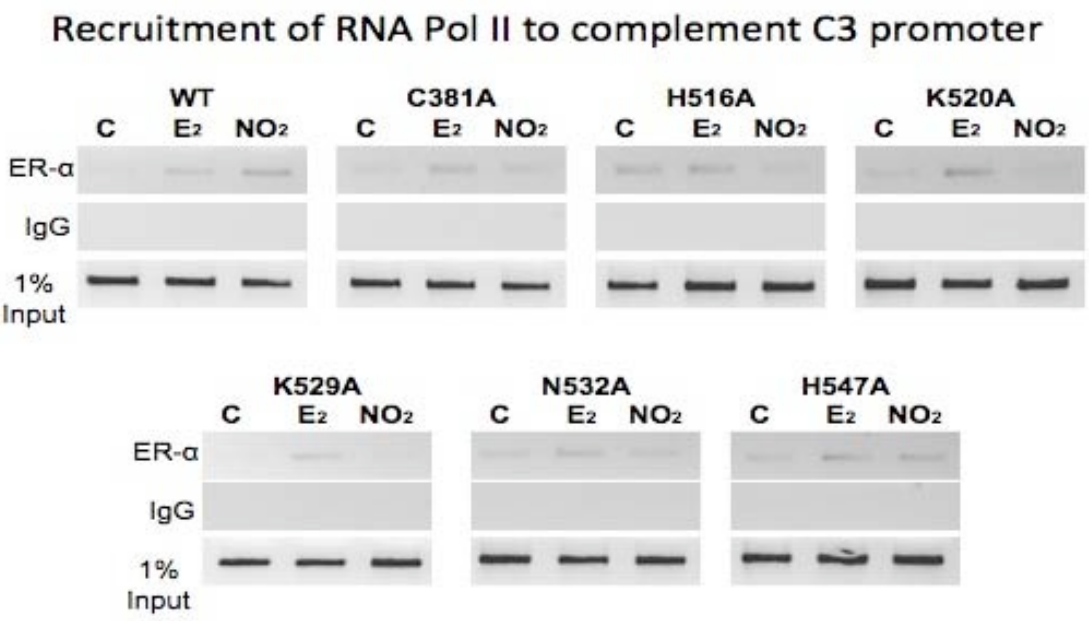

Figure 5. Effects of nitrite on the recruitment of RNA pol II to the hC3 promoter. ChIP assays were conducted as described above, however, following the first immunoprecipitation with ER-alpha antibody a second step of immunoprecipitation was performed with antibody to RNA pol II. PCR primers were used to amplify a region of the hC3 promoter.

In addition to visualizing the ChIP assays by standard ethidium bromide staining, I am currently working on using real-time PCR to provide more quantitative and sensitive results than end-point PCR. As can be seen in the results above, the bands for PCR are quite faint and hard to visualize. The results from the qPCR assays should also solve any of the background issues we have seen in some Re-ChIP assays. To date, I have designed specific qPCR primers for the hC3 and pS2 promoters and should conclude these studies within three months.

Key Research Accomplishments:

- Transfection
- ChIP and Re-ChIP assays
- Nitrate/Nitrite assay
- Co-immunoprecipitation
- qPCR
- Western blots

Reportable Outcomes:

- Poster Presentation Student Research Days March 2010, Georgetown University
- Poster Presentation Endocrine Society June 2010, San Diego

Conclusions

The work outlined in this report to date has shown that upon treatment with 1uM nitrite, wild-type ER-alpha dissociates from the hs90 complex, binds to DNA, and recruits SRC-1 and RNA polymerase II to estrogen regulated genes. Site A mutants lys529 and asn532 do not dissociate from the hsp90 complex, bind DNA and coactivator. Site B mutants, his516 and lys520 dissociate from the hsp90 complex; however, his516 did not bind DNA, while lys520 did. Finally, site C mutants dissociated from the hsp90 complex, cys381 did not bind to DNA, while his547 was recruited to DNA but did not bind coactivator. Inconsistent results from the asn532 mutant have been resolved since the previous annual report. I hope that future studies using immunohistochemistry will help to visualize the localization of wild type and mutant forms of ER-alpha after treatment with nitrite. In addition, the use of qPCR following ChIP and Re-ChIP assays will provide more quantitative and sensitive results to go along with the previous findings using standard PCR. Further, I look forward to studying the physiological interaction of ER-alpha with eNOS in MCF-7 breast cancer cells.

References

1. D.J. Veselik, S. Divekar, S. Dakshanamurthy, G.B. Storchan, J.M. Turner, K.L. Graham, L. Huang, A. Stoica and M.B. Martin, Activation of estrogen receptor-alpha by the anion nitrite, *Cancer Res.* **68** (2008), pp. 3950–3958

Appendices

Georgetown University Student Research Days 2010 Abstract

Activation of Estrogen Receptor-alpha by Nitrite

Geoffrey B. Storchan, Mary Beth Martin
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Recent studies suggest that nitrite is a novel second messenger that binds to and activates estrogen receptor-alpha (ER-alpha). Mutational analysis and molecular modeling identified three potential nitrite binding sites in the ligand binding domain (LBD). Site A is formed by lys529 on helix H11 and asn532 on the loop between helices H11 and H12; site B is formed by his516 on helix H10 and lys520 on helix H11; site C is formed by cys381 on helix H4 and his547 on helix H12. I hypothesize that nitrite activates ER-alpha by a three step mechanism. The model predicts that the interaction of nitrite with site A is necessary for the initial movement of helix H12 over the ligand binding pocket and dissociation of heat shock protein 90 (hsp90) from the receptor. Site B is involved in the straightening of helices H10 and H11, and is necessary for receptor dimerization. Finally, site C involving helices H4 and H12 is critical for the proper formation of the coactivator binding site of the receptor. I will test the ability of wild-type ER-alpha and binding site mutants for their ability to dissociate the hsp90 complex, to dimerize, to translocate into the nucleus and bind tightly to DNA, and to recruit coactivators.

Preliminary results demonstrate that wild-type ER-alpha dissociates from hsp90, is recruited to DNA, and binds coactivator when treated with nitrite. In addition, wild-type ER-alpha recruits coactivator SRC-1 and RNA polymerase II to DNA. The site C mutant, cys381, dissociates from hsp90, however, fails to bind DNA, or associate with coactivator upon nitrite treatment. Both site B mutants, his516 and lys520, dissociate hsp90, but only lys520 is recruited to DNA in the presence of nitrite, however, both fail to bind coactivator, suggesting they may play a role in forming the second step involving dimerization and binding of the receptor to DNA. Site A mutants, lys529 and asn532, do not dissociate from hsp90 or bind to DNA, therefore are critical in forming the initial step in nitrite activation. Site C mutant, his547, dissociates hsp90, binds to DNA and coactivator upon nitrite treatment, and as a result, may be critical in forming the final conformation necessary for coactivator recruitment.

Endocrine Society June 2010 Accepted Abstract Preview

Title: Activation of Estrogen Receptor-α by Nitrite

GB Storchan, MS, gbs7@georgetown.edu[1], KL Koenig klk38@georgetown.edu[1] and MB Martin, PhD, martinmb@georgetown.edu[1]. [1]Biochemistry and Molecular & Cellular Biology and Oncology, Georgetown University, Washington, DC, United States,20007.

Body: Recent studies show that the anion nitrite binds to and activates estrogen receptor-alpha (ER-alpha). Mutational analysis and molecular modeling identified three potential nitrite binding sites in the ligand binding domain (LBD) of the receptor. Site A is formed by lys529 on helix H11 and asn532 in the loop between helices H11 and H12; site B is formed by his516 on helix H10 and lys520 on helix H11; and site C is formed by cys381 on helix H4 and his547 on helix H12. To determine the mechanism by which nitrite activates the receptor, wild-type and mutants forms of ER-alpha were tested for their ability to dissociate from the heat shock protein 90 (hsp90) complex, translocate into the nucleus, dimerize and bind DNA, interact with coactivator, and recruit SRC-1 and RNA polymerase II. Preliminary results demonstrate that, upon treatment with 1uM nitrite, wild-type ER-alpha dissociated from hsp90, was recruited to DNA, bound coactivator, and recruited SRC-1 and RNA polymerase II to DNA. Site A mutants, K529A and N532A, did not dissociate from the hsp90-receptor complex, bind to DNA or bind coactivator. The site B mutants, H516A and K520A, dissociated from hsp90. However, H516A did not bind to DNA or recruit coactivator, while K520A bound to DNA, but did not recruit coactivator. The site C mutants, C381A and H547A, dissociated from hsp90. C381A failed to bind to DNA, while H547A was recruited to DNA but failed to bind coactivator. The results suggest a model whereby the interaction of nitrite with site A results in a conformational change at the interface of helix H11 and loop 11-12 that is necessary for the dissociation of hsp90. Site B is involved in the formation of a continuous helix between helices H10 and H11 that is necessary for binding to DNA. Site C, formed by amino acids located on helices H4 and H12, is involved in the recruitment to DNA as well as the formation of the coactivator binding site. Together, these findings represent a novel role for the anion nitrite in the activation of ER-alpha.

Sources of Support: National Institutes of Health R21ES015160-02 awarded to MBM DOD BCRP Predoctoral Traineeship Award BC073599 awarded to GBS